EMBO reports

scientific report

Snail genes at the crossroads of symmetric and asymmetric processes in the developing mesoderm

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Retinoic acid (RA) signalling ensures that vertebrate mesoderm segmentation is bilaterally synchronized, and corrects transient interferences from asymmetric left–right (L–R) signals involved in organ lateralization. *Snail* genes participate in both these processes and, although they are expressed symmetrically in the presomitic mesoderm (PSM), *Snail1* transcripts are asymmetrically distributed in the L–R lateral mesoderm. We show that the alteration of the symmetric *Snail* expression in the PSM induces asynchronous somite formation. Furthermore, in the absence of RA signalling, normal asymmetric *Snail1* expression in the lateral mesoderm is extended to the PSM, desynchronizing somitogenesis. Thus, Snail1 is the first cue corrected by RA in the PSM to ensure synchronized bilateral segmentation.

Keywords: Snail; retinoic acid; somitogenesis; left–right; pleiotropy

EMBO reports (2007) **8**, 104–109. doi:10.1038/sj.embor.7400867

INTRODUCTION

The bilateral symmetry in the body plan of vertebrate embryos is apparent in the somites, which are aligned in rows on either side of the neural tube. The periodic segmentation of the presomitic mesoderm (PSM) generates each pair of epithelial somites. The periodicity of this segmentation by reflected by regular pulses in the expression of components of the Notch and Wnt signalling pathways (Pourquié, 2003). These cycles of expression are symmetric in the left and right PSM, although how they are bilaterally synchronized remains unclear. In the absence of retinoic acid (RA), transient asymmetry is observed in vertebrate somite formation (Kawakami *et al*, 2005; Vermot *et al*, 2005; Vermot & Pourquié, 2005). Thus, during a short temporal window—the 'interference period'—symmetric somitogenesis is protected from left–right (L–R) asymmetric patterning cues by RA. However, the cues that RA buffers have not yet been identified.

Members of the Snail superfamily of transcription factors are expressed in distinct mesodermal territories, where they fulfil different roles (Nieto *et al*, 1994; Sefton *et al*, 1998; Carver *et al*, 2001). Owing to the high degree of modularity and reshuffling shown between *Snail* family members during vertebrate evolution (Locascio *et al*, 2002; Sefton *et al*, 1998), murine *Snail* and chicken *Slug* are the members expressed in the PSM (renamed *Snail1* and *Snail2*, respectively; Barrallo-Gimeno & Nieto, 2005). They seem to be functionally equivalent (del Barrio & Nieto, 2002) and the participation of one or the other in a particular process is determined by tissue-specific enhancers in each species (Locascio *et al*, 2002). In addition to their symmetrical expression in mesoderm territories, the right-hand lateral plate mesoderm (LPM) transiently expresses higher levels of *Snail1* than the left-hand side in both chick and mouse embryos (Sefton *et al*, 1998), reflecting its role in generating L–R asymmetry (Isaac *et al*, 1997).

Here, we show that the temporal window of L–R asymmetric expression of *Snail1* in the LPM coincides with the 'interference period'. *Snail* genes are expressed cyclically in the PSM, in which they integrate the Notch, Wnt and FGF signalling pathways and control somite epithelialization (Dale *et al*, 2006). We show here that their equivalent L–R levels in the PSM are necessary to maintain synchronic somitogenesis. Our data also show that RA blocks the asymmetric expression of *Snail1* in the PSM, preventing desynchronization and helping to discriminate between the territories in which Snail fulfils different roles.

RESULTS AND DISCUSSION

Asymmetric *Snail1* levels at the 'interference period'

Snail genes encode pleiotropic proteins that fulfil different functions during embryonic development and are simultaneously expressed in different mesodermal territories (Sefton *et al*, 1998). The expression of these genes in the PSM is cyclical, almost synchronous with genes of the Notch pathway and out of phase with *Axin2*—a cycling gene from the Wnt pathway (Dale *et al*, 2006). In addition, *Snail1* is transiently expressed asymmetrically in the LPM of chick and mouse embryos, where it influences organ lateralization (Isaac *et al*, 1997; Sefton *et al*, 1998). The bilateral

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Received 30 June 2006; revised 2 October 2006; accepted 10 October 2006; published online 24 November 2006

synchrony of somitogenesis is protected from the influence of organ lateralization during a short developmental window, known as the 'interference period'. We show that the transient L–R asymmetric expression of *Snail1* in the LPM occurrs at the 4–11 somite stage ($HH8^{-}HH10^{+}$) in chicken and mouse embryos (Fig 1A–D; data not shown), coinciding with the period in which RA offers protection from asymmetric signals.

In both species, the territories expressing *Snail1* are complementary to those with RA activity (Hochgreb *et al*, 2003; Vermot *et al*, 2005). Indeed, *Snail1* is expressed asymmetrically in regions devoid of RA activity, the LPM, where it is required for organ lateralization. Conversely, *Snail* gene expression is bilaterally symmetric in the anterior PSM where RA signalling is active. The inverse correlation between the sites of *Snail1* expression and RA signalling suggests that RA might regulate *Snail1* expression.

RA prevents asymmetric *Snail1* expression in the PSM

To determine whether RA signalling regulates asymmetric *Snail1* expression, chicken embryos were exposed to RA or disulphiram (DSM), an Raldh2 inhibitor, at stages when organ lateralization cues emanate from the node (Raya & Izpisua Belmonte, 2004). When analysed just before the interference period (the 4-somite stage; HH8), the asymmetric L–R expression of *Snail1* was lost in nearly 70% of the embryos treated with RA (18 out of 26; Fig 1E–H), indicating that RA signalling regulates *Snail1* asymmetric expression. However, this asymmetry in *Snail1* transcription was maintained in the presence of DSM (Fig 1I,J) and, as previously described, no alterations in bilateral synchronization were observed at this stage (Vermot & Pourquié, 2005).

Interestingly, when the embryos were analysed at the period of maximum interference (HH10), RA continued to abolish the asymmetric L–R *Snail1* expression in the LPM (four out of seven; Fig 1K–N,Q,R) without affecting the PSM (Fig 1U). By contrast, downregulation of RA signalling by DSM provoked the appearance of asymmetric L–R *Snail1* expression in the anterior PSM (three out of seven; Fig 1O,P,S) without affecting its expression in the LPM. Thus, RA administration exerted a strong influence in the LPM, a tissue devoid of endogenous RA signalling (Fig 1M,N,R), and DSM had a clear impact on the anterior PSM, a site of endogenous RA activity (Fig 1O,P,S). In DSM-treated embryos, both *Snail2* and *Lfng*, a cycling gene from the Notch pathway (McGrew *et al*, 1998), continued to cycle although their expression was asymmetric (Fig 1V–X). Indeed, diminished RA activity led to asymmetric somitogenesis as described previously (Fig 1X; Vermot & Pourquié, 2005). Thus, our data show that RA signalling regulates *Snail1* expression, and that when signalling is blocked *Snail1* is expressed asymmetrically in the PSM and asynchronous somitogenesis occurs.

Unilateral *Snail1* overexpression delays somitogenesis

We then investigated whether asymmetric *Snail* expression in the L–R PSM was sufficient to induce asynchronous somitogenesis. We misexpressed *Snail1* in one side of the chicken PSM through *in ovo* electroporation and, as when RA signalling was abolished in chick embryos (Vermot & Pourquié, 2005), increased expression of Snail1 in one side desynchronized somite formation ($n = 15$; Fig 2A–H). Although *Snail2* continued cycling (data not shown), somitogenesis was delayed in the side with increased *Snail1* expression in two-thirds of the embryos, as assessed by

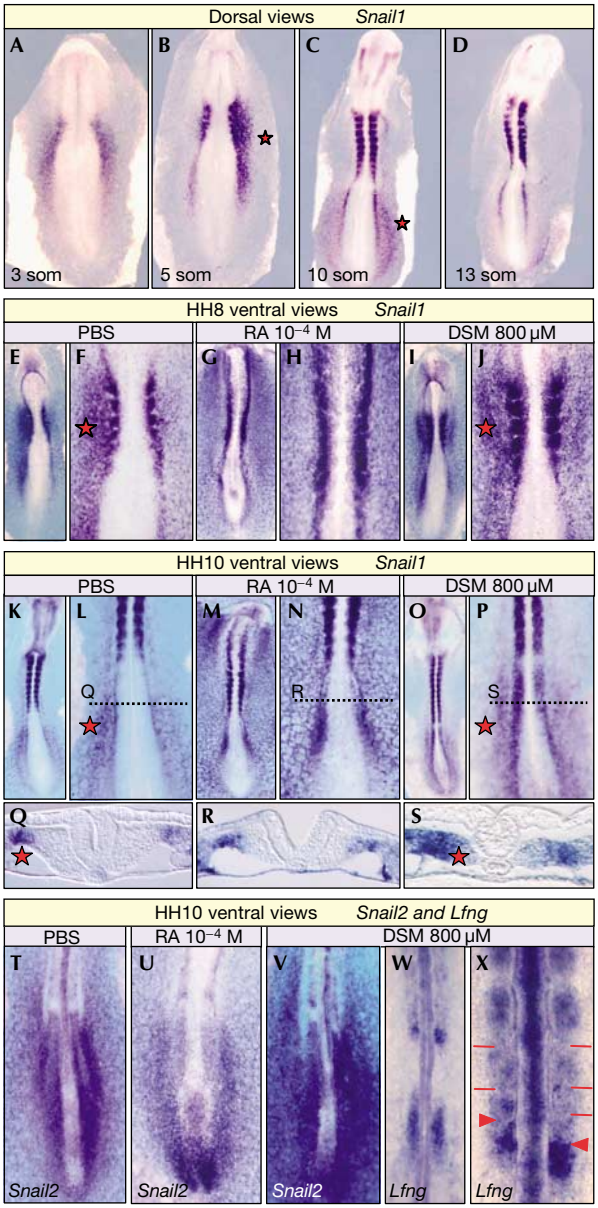

Fig 1 | Retinoic acid signalling prevents asymmetric *Snail1* expression in the anterior presomitic mesoderm. (**A–D**) Transient asymmetric left–right (L–R) *Snail1* expression in the lateral plate mesoderm (LPM) of 4- to 11-somite (som) chicken embryos (Isaac *et al*, 1997). (**E–X**) Embryos incubated with PBS, retinoic acid (RA) or disulphiram (DSM) and analysed at the 4-somite (HH8; **E–J**) or 10-somite stage (HH10; **K–X**). (**Q**), (**R**) and (**S**) are sections through the LPM at the levels indicated in (**L**), (**N**) and (**P**), respectively. Embryos were hybridized for *Snail1* (**A–S**), *Snail2* (**T–V**) and *Lfng* (**W,X**). Exposure to RA abolishes asymmetric L–R *Snail1* expression in the LPM (**G,H,M,N**) without affecting *Snail2* expression in the presomitic mesoderm (PSM; **T,U**). Asymmetric L–R *Snail1* expression invades the anterior PSM in HH10 embryos treated with DSM (**O,P,S**), which delayed segmentation on the side of highest *Snail1* expression (**X**). Asymmetric L–R expression in the LPM is not affected in these embryos (**P**), where both *Snail2* and *Lfng* continue cycling in the PSM (**V–X**). Red stars indicate asymmetric L–R expression, red bars the somite boundaries and red arrowheads the newly formed somite boundaries.

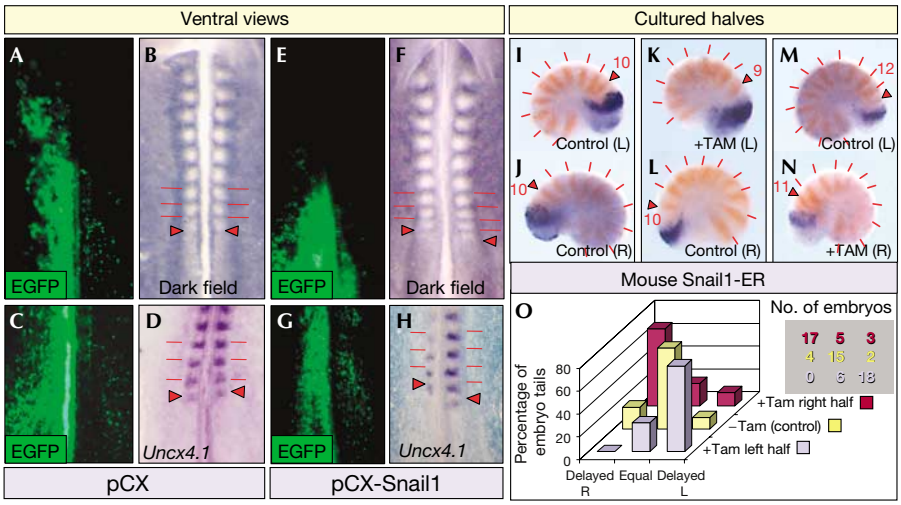

Fig 2 | Unilateral Snail1 overexpression delays somite formation in chick and mouse embryos. Expression of EGFP (**A,C,E,G**), the morphology (**B,F**) and *Uncx4.1* expression (**D,H**) in chicken embryos electroporated with the EGFP expression vector (pCX) (**A–D**) or pCX-Snail1 (**E–H**). Caudal halves of 10.5 dpc Snail1-ER transgenic mouse embryos cultured for 15 h in the presence (**K,N**) or absence (**I,J,L,M**) of 4′-OH-tamoxifen and analysed for *Lfng* (blue), and *Uncx4.1* (orange) expression. Red bars indicate somite boundaries and red arrowheads the newly formed somite boundaries. (**O**) Diagram quantifying the percentage of embryos that showed synchronous or asynchronous somitogenesis after the different conditions in culture. Delayed R, embryos with delayed somitogenesis on the right side; Equal, embryos showing synchronic bilateral somitogenesis; Delayed L, embryos with delayed somitogenesis on the left. The number of embryos represented is also shown in a table that maintains the same colour code and relative position. dpc, days postconception.

morphology and *Uncx4.1* expression (10 out of 15; Fig 2E–H), whereas somite formation progressed synchronously in embryos electroporated with control constructs ($n=8$; Fig 2A–D). These data indicate that bilateral asymmetric expression of Snail1 is sufficient to induce desynchronization. Thus, it should be avoided in the chick PSM to maintain bilateral synchrony.

In the mouse, *Snail1* is also asymmetrically expressed in the LPM during the 'interference period' (Sefton *et al*, 1998), but unlike in the chick, it is the family member that cycles synchronously in the L–R PSM (Dale *et al*, 2006). Thus, in the mouse, the expression of the *Snail* genes in the LPM and PSM is associated with *Snail1*. The asynchronous somitogenesis observed in the absence of RA signalling in the mouse (Vermot *et al*, 2005) suggests that the requirement for bilateral symmetrical *Snail1* expression is evolutionarily conserved. However, because this remains to be shown, we generated a transgenic mouse carrying a tamoxifen-inducible form of Snail1 (hereafter Snail1 transgenic; see Methods and supplementary figure online). We cultured bisected caudal regions of Snail1 transgenic embryos 10.5 days postconception (dpc; $n=70$; Fig 2I–N). When the two halves of these embryos were cultured in medium alone ($n=21$), we observed asymmetric *Lfng* expression in less than one-third of the embryos (6 out of 21; Fig 2I,J,O), and there were no discrepancies in somite number in any of the embroys. By contrast, when one-half was cultured in the presence of tamoxifen and the other half was maintained in a control medium ($n=49$), somitogenesis was delayed in the half that were tamoxifen-treated (35 out of 49, 71%; Fig 2O); there was one somite less compared with the control half (Fig 2K–N). Out of the 49 embryo tails, in which Snail1 was specifically activated in one-half, 25 were right halves (shown in brown in Fig 2O) and 24 were left halves (shown in light purple in Fig 2O). Snail1 overexpression in either the left or right side produced similar results (Fig 2O). These data indicate that this effect was not lateralized and confirm that, as in the chick, increasing the levels of Snail1 expression in one side of the PSM causes a delay in somite formation.

Symmetric somitogenesis requires equal L–R Snail levels

As discussed earlier, and in contrast to the mouse, a Snail family member is expressed in the chick PSM. As they are thought to be functionally equivalent when expressed in similar territories, we checked whether increasing the levels of Snail2 expression in one side of the PSM would induce the same effects as Snail1. When control vectors were electroporated in one side of the embryonic PSM ($n=10$; Fig 3A,B), or when similar levels of Snail2 were misexpressed in both sides of the PSM ($n=18$; not shown), there was no clear effect on synchronization. By contrast, unequal misexpression of Snail2 in the left or right somitic mesoderm disrupted somite alignment (17 out of 34), with 66% of these embryos developing fewer somites in the side with higher levels of Snail2 (11 out of 17; Fig 3F,G). We analysed these asymmetries by defining the expression of *Lfng* and *Hairy2*, another cycling gene from the Notch pathway (Jouve *et al*, 2000), in embryos overexpressing Snail2 or a dominant-negative Snail2 construct lacking the zinc-fingers (Δ*Zf-Snail2*; Aybar *et al*, 2003). Expression of the cycling genes was always bilaterally symmetrical in electroporated control embryos (ten out of ten for *Lfng* and seven out of seven for *Hairy2*; Fig 3C,D). By contrast, this symmetry was disrupted by unequal L–R expression in the PSM of either *Snail2* (72%, 18 out of 25 for *Lfng* and 64%, 7 out of 11 for Hairy2; Fig 3H,I) or its dominant-negative form (61%, 14 out of 23 for *Lfng* and 47%, 8 out of 17 for *Hairy2*; Fig 3M,N). Like Snail1

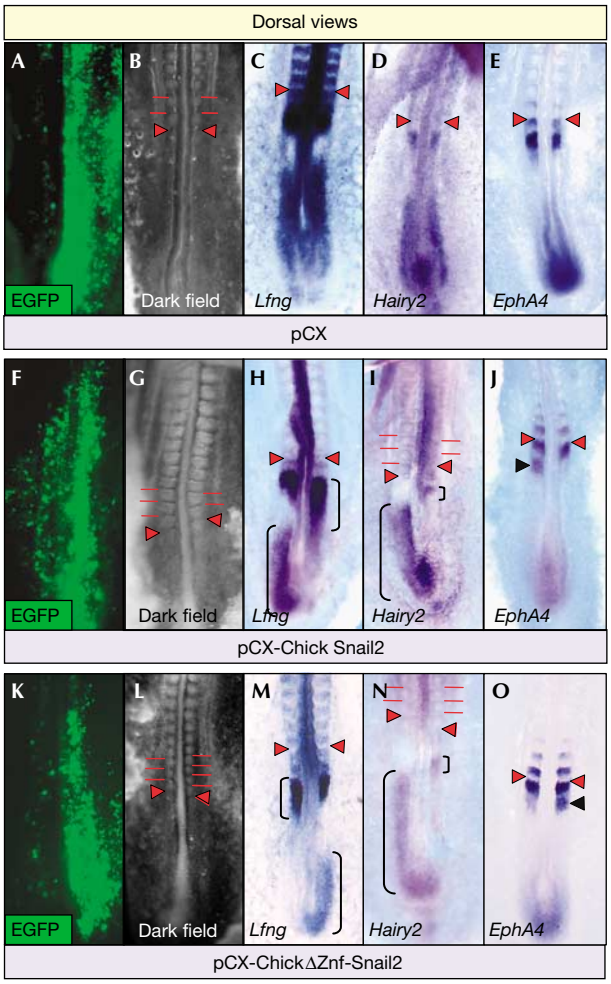

Fig 3 | Delayed or accelerated somite formation provoked by Snail2 overexpression or dominant–negative Snail2 expression in the chick. Chicken embryos electroporated with pCX-dEGFP plus pCX (**A–E**), pCX-Snail2 (**F–J**) or a dominant-negative form of *Snail2* (pCX-ΔZf-Snail2; **K–O**) showing dEGFP expression (**A,F,K**), their morphology (**B,G,L**) and the expression of *Lfng* (**C,H,M**), *Hairy2* (**D,I,N**) and *EphA4* (**E,J,O**). High levels of ectopic expression were observed in the right presomitic mesoderm (PSM) of the embryos. Brackets indicate the progress of PSM expression. The asymmetric phase of the cycling genes represented in this figure corresponds to embryos in which the differences were more apparent. Red bars indicate somite boundaries, red arrowheads the newly formed boundaries and black arrowheads indicate an extra band of *EphA4* expression.

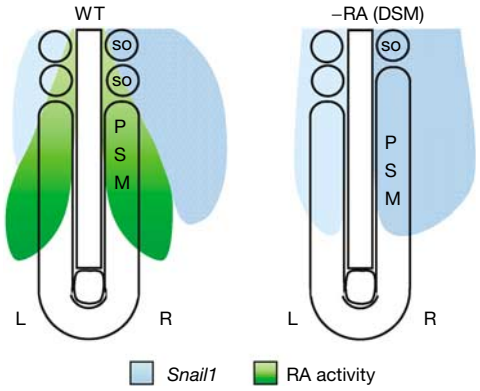

Fig 4 | Inhibition of retinoic acid signalling provokes asymmetric *Snail1* expression in the presomitic mesoderm inducing desynchronization in somite formation. Diagram depicting RA signalling activity and *Snail1* expression in the wild-type embryo and after inhibiting RA signalling. Endogenous RA prevents the invasion of the left–right (L–R) asymmetric Snail1 expression in the PSM and ensures synchronic somitogenesis. DSM, disulphiram; PSM, presomitic mesoderm; RA, retinoic acid; WT, wild type.

overexpression in the mouse, delayed expression occurred in the side exhibiting higher *Snail2* expression (83%, 15 out of 18 for *Lfng* and 86%, six out of seven for *Hairy2*). The delay in segmentation was also similar to that obtained after increasing Snail1 expression (Fig 2A–H), again reflecting that the two Snail proteins are functionally equivalent when expressed in similar territories (del Barrio & Nieto, 2002; Bolos *et al*, 2003). Interestingly, somitogenesis was more advanced in the side of the PSM with higher level of Δ*Zf-Snail2* (81%, 18 out of 22), and an extra somite developed in one-third of these embryos (Fig 3K–N). Thus, the phenotype induced by expressing a Snail2 dominant-negative form was complementary to that found after Snail2 overexpression.

The *EphA4* receptor is a marker of somite epithelialization and boundary formation (Barrios *et al*, 2003), and its symmetric expression (10 out of 11; Fig 3E) was also disrupted by these constructs. One stripe was lost in the side with higher *Snail2* expression (7 out of 13; Fig 3J), whereas an extra stripe appeared in the side with higher levels of Δ*Zf-Snail2* (three out of nine; Fig 3O). Our data from studies on chick and mouse embryos confirm that *Snail* genes regulate somite boundary formation and that increased Snail activity delays epithelialization. This is in agreement with recent data showing that the downregulation of *Snail* genes in the anterior PSM determines the time of epithelialization (Dale *et al*, 2006), and the role of Snail in maintaining the mesenchymal phenotype of undifferentiated cells (reviewed by Nieto, 2002).

Significantly, we observed the same phenotypes in embryos electroporated at different developmental times, up to the 30-somite stage (HH16). Thus, regardless of the role of the cyclical expression of *Snail* genes in the posterior mesoderm and in epithelialization, equivalent L–R levels of *Snail* expression in the PSM are necessary to maintain synchronous bilateral segmentation during somitogenesis in both chick and mouse embryos. This reflects the requirement that the L–R asymmetric Snail1 expression is excluded from the anterior PSM and also explains the temporal coincidence of the 'interference period' with the asymmetric L–R *Snail1* expression in the embryo. Before this period, the inhibition of RA signalling does not have any effect on somite synchronization or Snail1 expression. At the period of maximal interference, when RA signalling is inhibited, asymmetric *Snail1* expression develops in the anterior PSM and induces desynchronization. In conclusion, endogenous RA activity in the anterior PSM acts as a barrier that prevents the entry of the asymmetric Snail1 expression directed by the L–R patterning

signals in the region where somite boundaries form, thereby ensuring bilateral symmetry during somitogenesis (Fig 4).

The *Snail* gene family is a good example of pleiotropic genes that might cause interferences when the different developmental processes in which they are involved occur simultaneously in adjacent or overlapping regions. Under these circumstances, correction mechanisms, such as bilaterally symmetric somitogenesis and asymmetric organ lateralization, are required when the processes conflict.

METHODS

Embryo dissection and *in situ* hybridization. Chicken and mouse embryo staging, and the method for caudal bilateral dissections and explant culture are described by Morales *et al* (2002). Whole-mount *in situ* hybridization was carried out as described by Sefton *et al* (1998) by using the chick *Snail1, Snail2*, *Lfng* and *Lfng* intronic, *Hairy2*, *EphA4* and *Uncx4.1*, and the mouse *Snail1*, *Lfng* and *Uncx4.1* riboprobes (Irving *et al*, 1996; Sefton *et al*, 1998; Jouve *et al*, 2000; Morales *et al*, 2002; Uncx4.1, BBSRC chicken clone ChEST47F8). In some cases, 40 μm vibratome sections were used from gelatin-embedded embryos.

Plasmids. The EGFP expression vector (pCX-dEGFP) contains a destabilized EGFP construct (d1EGFP, Clontech, Mountain View, CA, USA) with a half-life of approximately 1 h in the pCAAGS expression vector (Niwa *et al*, 1991). The full-length chicken *Snail1* and *Snail2* coding sequences (Sefton *et al*, 1998) and a truncated *Snail2* construct (aa 1–134) were cloned into the pCAAGS expression vector (pCX-*Snail1/2* and pCX-ΔZf-*Snail2*, respectively). These pCX plasmids were electroporated at concentrations of 3 μg/μl with 1 μg/μl of pCX-EGFP. The empty expression vector and pCX-dEGFP were electroporated into control embryos. The fusion protein between Snail1 and a modified human oestrogen receptor (pCMVSnail1-ERT2) was generated by cloning the mouse Snail1 coding region into the pCre-ERT2 expression vector (a generous gift from Dr Pierre Chambon of Feil *et al*, 1997) before transferring it into pcDNA3.

***In ovo* electroporation and chicken embryo culture.** Stage HH5 embryos were electroporated as described previously (Dubrulle *et al*, 2001). A train of electric pulses (six pulses, 30 V, 50 ms) was applied by using a square wave electroporator (Intracel TSS20). Embryos were left for 20–40 h (mostly 30 h) and assayed for dEGFP expression. Embryos with a normal overall morphology and good levels of EGFP expression in the PSM were processed for *in situ* hybridization. Chicken embryos were explanted at stage HH4 and cultured as described by Chapman *et al* (2001). Where appropriate, the embryos were exposed to 100 μl of RA (100 μM) and DSM (800 μM) in PBS and the treated embryos were processed for *in situ* hybridization.

Transgenic mice, PSM culture and tamoxifen induction. A transgenic mouse for pCMV-Snail1-ERT2 was generated (Hogan *et al*, 1994) in which the constitutively expressed protein is only functional when translocated into the nucleus upon tamoxifen administration (see supplementary figure online). Bisected 10.5 dpc caudal halves (PSM plus three somites) were cultured for 15 h as described by Morales *et al* (2002), with 50 μg/ml of gentamycin (Gibco, Carlsbad, CA, USA) in the culture medium. In culture, 6–7 new somites were formed and, where appropriate, the cultured halves were exposed to 600 nM 4-OH-tamoxifen (Sigma, St Louis, MO, USA; Feil *et al*, 1997).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank M. Torres and J. Galceran for comments on the manuscript; B. Lázaro, J. Chuliá and C. López for technical assistance; K. Dale for assistance with electroporation; and M. Okabe, J. Miyazaki, O. Pourquié, H. Peters and P. Chambon for reagents. This work was funded by grant BFU2005-00762 to A.V.M., and grants BFU2004-02665, BFU2005-05772 and NAN2004-09230-C04-04 to M.A.N. C.A.F. was supported by BFU2004-02665 and NAN2004-09230-C04-04, A.V.M. by Advancell S.L. and the Ramon y Cajal Programs. A.V.M. and H.A. were also supported by the I3P Programme (European Social Fund/MEC), H.A. and O.H.O. by Spanish Ministry fellowships and V.G. by the Institute of International Cooperation.

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